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A Variable Cluster of Ethylene Response Factor–Like Genes Regulates Metabolic and Developmental Acclimation Responses to Submergence in Rice [W]

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***Submergence*-1 (*Sub*1), a major quantitative trait locus affecting tolerance to complete submergence in lowland rice (*Oryza sativa*), contains two or three ethylene response factor (ERF)–like genes whose transcripts are regulated by submergence. In the submergence-intolerant *japonica* cultivar M202, this locus encodes two ERF genes, *Sub*1B and *Sub*1C. In the tolerant near-isogenic line containing the *Sub*1 locus from the *indica* FR13A, M202(*Sub*1), the locus additionally encodes the *ERF* gene *Sub*1A. During submergence, the tolerant M202(*Sub*1) displayed restrained leaf and internode elongation, chlorophyll degradation, and carbohydrate consumption, whereas the enzymatic activities of pyruvate decarboxylase and alcohol dehydrogenase were increased significantly compared with the intolerant M202. Transcript levels of genes associated with carbohydrate consumption, ethanolic fermentation, and cell expansion were distinctly regulated in the two lines. *Sub*1A and *Sub*1C transcript levels were shown to be upregulated by submergence and ethylene, with the *Sub*1C allele in M202 also upregulated by treatment with gibberellic acid (GA). These findings demonstrate that the *Sub*1 region haplotype determines ethylene- and GA-mediated metabolic and developmental responses to submergence through differential expression of *Sub*1A and *Sub*1C. Submergence tolerance in lowland rice is conferred by a specific allele variant of *Sub*1A that dampens ethylene production and GA responsiveness, causing quiescence in growth that correlates with the capacity for regrowth upon desubmergence.**

INTRODUCTION

Flooding of croplands is a frequent natural disaster in many regions of the world. The flooding of root systems and partial to complete submergence of aerial organs can dramatically reduce crop productivity. Plant submergence attributable to complete flooding restricts the diffusion of oxygen and carbon dioxide by 10^4-fold, which has a dramatic impact on biochemical activities, such as aerobic respiration and photosynthesis (Armstrong and Drew, 2002). The inhibition of these processes stimulates a variety of responses that can enhance the survival of transient inundation. Acclimative responses are species and genotype specific and can include altered rates of petiole/internode elongation, altered anatomy and cell ultrastructure in leaves and roots, development of lateral/adventitious roots, formation of aerenchyma, and a switch from aerobic to anaerobic respiration (Drew, 1997; Fukao and Bailey-Serres, 2004). The acclimative responses to submergence stress also engender potentially lethal side effects, such as carbohydrate starvation and the accumulation of toxic end products that acidify the cytosol or damage membrane integrity (Drew, 1997; Gibbs and Greenway, 2003; Fukao and Bailey-Serres, 2004).

Most nonaquatic plants are damaged by transient inundation of aerial tissue for 24 to 48 h. Exceptionally, rice (*Oryza sativa*) is generally tolerant of submergence. Deepwater rice and the widely cultivated lowland rice overcome submergence stress by antithetical strategies (Fukao and Bailey-Serres, 2004). Deepwater rice avoids the stress by the activation of gibberellic acid (GA)–promoted internode elongation, which allows plants to outgrow submergence and thereby restore gas exchange above the water surface. Lowland rice cultivars are typically cultivated in flooded paddies but are generally intolerant of complete submergence. The submergence-tolerant East Indian accession FR13A restricts leaf and internode elongation during inundation and can recommence the initiation of leaf development upon desubmergence (Singh et al., 2001; Das et al., 2005). Physiological responses to submergence stress in rice have been compared using tolerant and intolerant varieties. However, none of these analyses has been performed on near-isogenic lines. Consequently, the significance and genetic determinants of the observed variation in metabolic and developmental responses have remained unclear.

Submergence-1 (*Sub*1) is a major quantitative trait locus affecting submergence tolerance in lowland rice, which accounts for 35 to 69% of phenotypic variance in tolerance in diverse backgrounds (Xu and Mackill, 1996; Nandi et al., 1997; Sripongpangkul et al., 2000; Xu et al., 2000; Toojinda et al., 2003). Detailed genetic and physical mapping of *Sub*1 revealed that this

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locus contains a variable cluster of two to three genes that encode proteins with the DNA binding domain common to the ethylene response factors (ERFs)/ethylene-responsive element binding proteins/Apetala2-like proteins (Xu et al., 2006). All three *Sub*1 region genes fall in the B-2 subclass of ERF proteins, which contains a single 58- to 59-residue ERF domain. It has been shown in a few cases to interact with GC-rich *cis*-acting sequences of target genes and to regulate ethylene-mediated responses (Gutterson and Reuber, 2004). The genes *Sub*1B and *Sub*1C are present in a wide range of *indica* and *japonica* varieties, whereas *Sub*1A is limited to a subset of *indica* varieties. A single amino acid substitution distinguishes the *Sub*1A-1 allele of tolerant *indica* varieties, including FR13A. The *Sub*1A alleles in tolerant and intolerant varieties are highly and poorly induced under submergence, respectively. Moreover, *Sub*1A is absent from all *japonica* germplasms studied and some *indica* germplasms, all of which are submergence-intolerant (Xu et al., 2006). In support of *Sub*1A as a key determinant of submergence tolerance, the transformation of the intolerant *japonica* variety Liaogeng with a constitutively expressed *Sub*1A gene significantly increased tolerance to submergence (Xu et al., 2006).

Submergence promotes the biosynthesis of 1-aminocyclopropane-1-carboxylic acid, which is converted to ethylene in an oxygen-dependent manner (Banga et al., 1996; Kende et al., 1998; Peng et al., 2001). Oxygen produced by photosynthesis or from the surrounding aqueous environment can be used for ethylene synthesis during submergence (Mommer et al., 2004, 2005). Ethylene levels can also increase as a result of physical entrapment by the surrounding aqueous environment, as observed in the semiaquatic eudicot *Rumex palustris* (Banga et al., 1996). This hormone plays important roles in the acclimative responses to submergence and flooding. It contributes to the programmed cell death that leads to the formation of aerenchyma and adventitious roots under hypoxia (Drew et al., 2000; Mergemann and Sauter, 2000; Gunawardena et al., 2001) and promotes internode and petiole elongation that allows deepwater rice and *R. palustris* to extend photosynthetic organs above the water surface (Kende et al., 1998; Peeters et al., 2002). Cell elongation under submergence involves increased accumulation of gene transcripts encoding expansins, which mediate cell wall loosening. In *R. palustris*, *Expansin A* transcripts increased after submergence and ethylene treatment, and the induction by submergence was suppressed by an ethylene action inhibitor, 1-methylcyclopropene (Vreeburg et al., 2005). Ethylene also triggers an increase in the endogenous content of GA and the sensitivity to this phytohormone in deepwater rice and *R. palustris*, leading to the conclusion that internode and petiole elongation during submergence results from ethylene-mediated GA responses (Kende et al., 1998; Peeters et al., 2002). Recently, it was shown that the ethylene entrapped during submergence contributed to a decrease in endogenous abscisic acid content in *R. palustris* (Benschop et al., 2005). The change in both of these hormones was a prerequisite for petiole elongation.

In this study, we compared the acclimative responses to submergence of near-isogenic *japonica* lines that differ in haplotype of an ERF gene cluster at the *Sub*1 locus. Our results demonstrate that the ERF domain genes *Sub*1A and *Sub*1C are differentially regulated by submergence, ethylene, and GA at the level of transcript accumulation. The presence of *Sub*1A-1 at the *Sub*1 locus results in ethylene- and GA-mediated negative regulation of genes associated with carbohydrate catabolism and cell elongation as well as positive regulation of genes involved in ethanolic fermentation during submergence. These data demonstrate that evolutionary divergence in an ERF domain gene cluster is responsible for the positive and negative gene regulation that controls multifaceted cellular and developmental responses to submergence and confers submergence tolerance in lowland rice.

RESULTS

Regulation of Three *ERF* Genes at the *Sub*1 Locus under Submergence

The *Sub*1 region on rice chromosome 9 contains a cluster of two or three *Sub*1 genes (*Sub*1A, *Sub*1B, and *Sub*1C), and genotypic variation at this complex locus confers distinctions in submergence tolerance (Xu et al., 2006). Submergence-intolerant *japonica* rice, including the inbred M202, lacks *Sub*1A and possesses *Sub*1B and *Sub*1C. The near-isogenic line M202(*Sub*1) was generated by introgression of the *Sub*1 region from the submergence-tolerant *indica* cultivar FR13A (Xu et al., 2004). The M202(*Sub*1) introgression line contains all three *Sub*1 genes of *indica* origin in a genomic region of 182 kb based on molecular mapping and genomic sequence analysis (Figure 1A) (Xu et al., 2006). In the submergence stress studies presented here, 14-d-old plants were completely submerged for up to 14 d and leaf tissue was collected at six time points. Semiquantitative RT-PCR analysis confirmed the absence of the *Sub*1A transcript in leaves of M202, consistent with the absence of this gene from the Nipponbare genome sequence and other *japonica* varieties studied. The level of *Sub*1A mRNA increased rapidly in abundance after 1 d of submergence in M202(*Sub*1) leaves and remained increased for up to 14 d of stress (Figure 1B). The *Sub*1B transcript was detected in both genotypes and was slightly upregulated by the stress, with higher levels of the transcript consistently detected in M202(*Sub*1). As observed for *Sub*1A, the *Sub*1C transcript was increased dramatically for the duration of the stress period. The level of the *Sub*1C transcript was higher in M202 compared with M202(*Sub*1). The distinctions in *Sub*1B and *Sub*1C mRNA levels under stress in the two lines may be attributable to the distinct regulation of the *japonica* versus *indica* alleles or may reflect the absence versus presence of *Sub*1A in M202 and M202(*Sub*1), respectively.

Submergence Recovery and Chlorophyll Maintenance under Submergence Are Influenced by the *Sub*1 Haplotype

Fourteen-day-old plants of these near-isogenic lines consisted of a coleoptile and two fully expanded leaves with no evident difference in leaf and internode development. The influence of the M202 versus the FR13A *Sub*1 haplotype on submergence tolerance and the viability of the coleoptile, leaf, and whole plant was evaluated at 7 d after recovery from 7 or 14 d of submergence (Figures 2A and 2B). The two genotypes had a distinct appearance upon desubmergence. M202 leaves were more

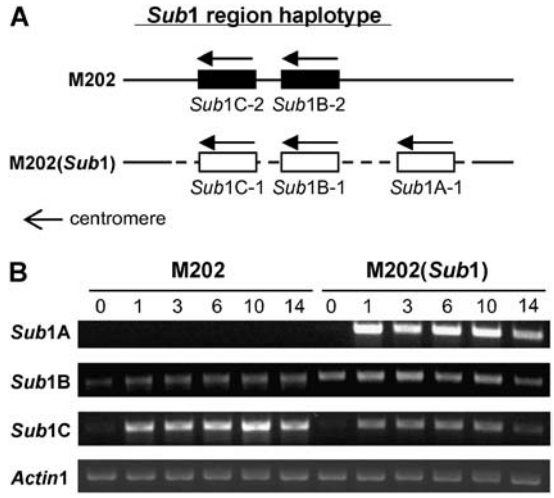

Figure 1. Comparison of the Allele Composition and mRNA Accumulation of *Sub*1 Region Genes in M202 and M202(*Sub*1).

(A) Gene and allele composition of ERF domain genes in the *Sub*1 region of chromosome 9. The *Sub*1 haplotype in M202 (*japonica*) consists of *Sub*1B-2 and *Sub*1C-2, whereas the genomic region in M202(*Sub*1) encodes *Sub*1A-1, *Sub*1B-1, and *Sub*1C-1 (Xu et al., 2006). The dashed line indicates the ~182 kb introgressed from the *indica* accession FR13A. Arrows represent the direction of transcription for the ERF genes.
(B) Analysis of *Sub*1 gene transcript accumulation in M202 and M202(*Sub*1) leaves during submergence. Fourteen-day-old plants were submerged for up to 14 d, and leaf tissue was harvested at specific time points (days 0, 1, 3, 6, 10, and 14). Total RNA was analyzed by semiquantitative RT-PCR using gene-specific primers for *Sub*1A, *Sub*1B, and *Sub*1C. The level of *Actin*1 mRNA was used as a loading control. The number of cycles for linear amplification was optimized for each primer pair. Representative results from at least three independent biological replicate experiments are shown.

prostrate, spindly, and chlorotic. After 7 d of submergence, only 30.7% of the second leaves of M202 were intact, whereas all leaves of M202(*Sub*1) were fully green. The viability of the coleoptile and first leaf was not significantly different in the two genotypes after 7 d of inundation. After a more prolonged submergence (14 d), all of the first and second leaves of M202 appeared brown and withered, whereas all of the first leaves and 81.3% of the second leaves of M202(*Sub*1) were fully green and appeared undamaged. The coleoptile of M202(*Sub*1) was also considerably more tolerant to 14 d of submergence. Viability after 14 d of submergence was significantly different in these lines, as judged by the emergence and growth of the third and subsequent leaves (Figure 2B). All of the M202(*Sub*1) plants produced new leaves during the recovery period, whereas only 32.0% of the M202 plants did so. These data confirm that the introgression of the FR13A *Sub*1 region into M202 is sufficient to dramatically enhance the viability of fully emerged leaves and to confer the ability to resume apical meristem development upon desubmergence.

In rice, submergence stress increases the transcript level and enzymatic activity of chlorophyllase, which promotes the degradation of chlorophyll (Ella et al., 2003). To determine whether genetic variation at the *Sub*1 region influences chlorophyll maintenance (biosynthesis and/or breakdown) during submergence, chlorophyll contents were monitored over 14 d of submergence (Figure 2C). Chlorophyll *a*/*b* contents declined gradually in both genotypes, but M202(*Sub*1) maintained significantly higher levels of chlorophyll than did M202 from day 6. Chlorophyll *b* content decreased more rapidly than chlorophyll *a* content, reflecting the conversion of chlorophyll *b* to *a* in the degradation pathway (Folly and Engel, 1999). These data confirm that the FR13A *Sub*1 haplotype enhances the maintenance of chlorophyll during submergence.

Differential Regulation of Elongation under Submergence Is Controlled by the *Sub*1 Haplotype

Generally, submergence-sensitive lowland rice cultivars elongate more rapidly than tolerant cultivars under submergence stress (Singh et al., 2001; Das et al., 2005). To examine whether the *Sub*1 haplotype controls growth rate, plant height was compared in M202 and M202(*Sub*1) plants grown under normal (aerobic) or submerged conditions for 14 d (Figure 3A). The height of 14-d-old plants of these genotypes was nearly identical, and both lines grew uniformly under normal conditions. In response to 14 d of submergence stress, M202 displayed significantly greater elongation than M202(*Sub*1). The increase in plant height of the tolerant line was the same under submergence and normal conditions, indicating an acceleration in elongation of the intolerant line under the stress. These data confirm that the FR13A *Sub*1 haplotype suppresses elongation during submergence but does not influence plant height under normal conditions. At maturity, these two lines were indistinguishable in height (data not shown). The lack of effect of the *Sub*1 haplotype on normal growth and development is consistent with the observation that the *Sub*1A gene was submergence-inducible.

Expansins are proteins that mediate cell wall loosening in plants. Increased expansin mRNA and protein levels are correlated with cell elongation (Li et al., 2003). A total of 80 expansin-encoding genes have been recognized in the *japonica* rice genome and classified into four subgroups by phylogenetic analysis (Li et al., 2003). At least seven *Expansin A* (*ExpA*) mRNAs accumulate in leaves of deepwater rice, and their abundance was upregulated by submergence (Lee and Kende, 2002). The levels of five *ExpA* transcripts, *ExpA*1, *ExpA*5, *ExpA*6, *ExpA*7, and *ExpA*16, were assessed by semiquantitative RT-PCR analysis in the two near-isogenic lines (Figure 3B). All five mRNAs, except for *ExpA*7, were gradually increased in M202 over the 14 d of submergence. By contrast, in M202(*Sub*1), these transcripts increased slightly until day 3 or 6 and then declined. A higher and more sustained accumulation of *ExpA* mRNAs during submergence was consistent with the significant promotion of leaf and internode elongation in the intolerant M202.

Carbohydrate Consumption under Submergence Is Modulated by the *Sub*1 Haplotype

Plant cells consume carbohydrates through energetically inefficient coupling of glycolysis and anaerobic fermentation when oxygen levels are restricted (Drew, 1997). An additional consequence of submergence is that carbohydrate production

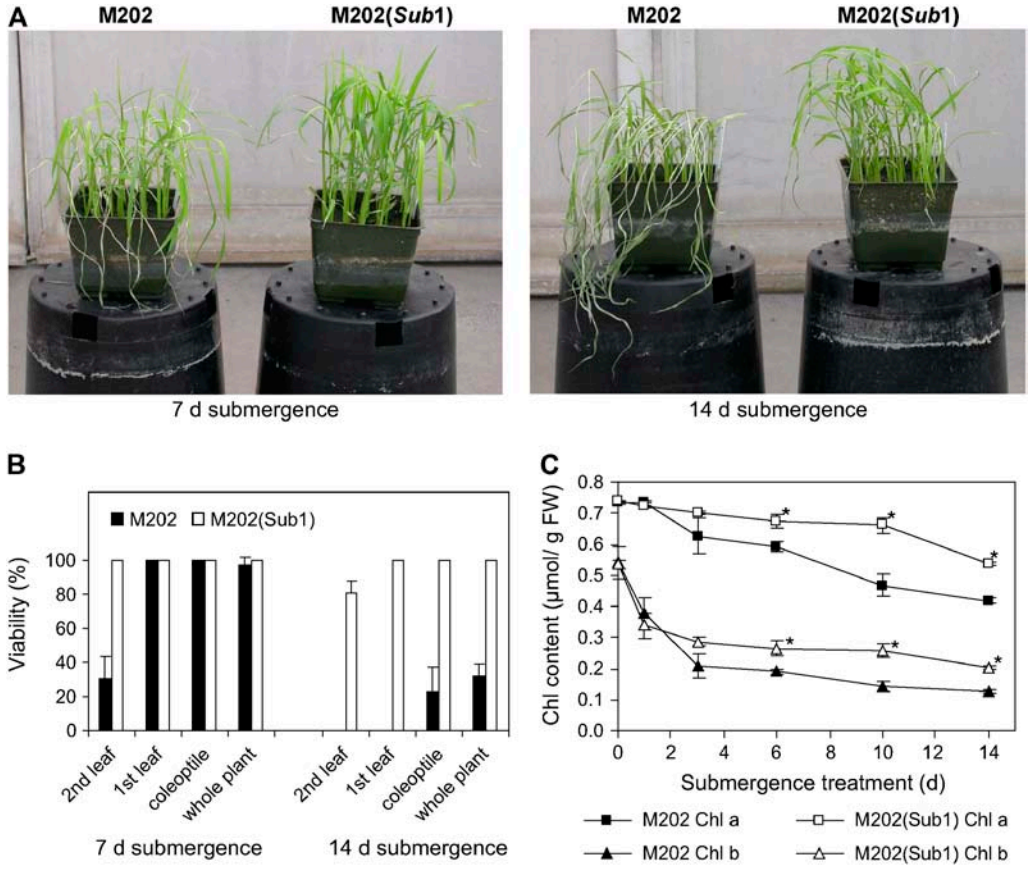

Figure 2. Phenotypes of M202 and M202(*Sub*1) Plants after Submergence.

(A) Rice plants after 7 d of recovery from submergence. Fourteen-day-old plants were submerged for 7 d (left) or 14 d (right). After submergence, plants were returned to normal growth conditions for 7 d and photographed.

(B) Viability of coleoptile, first leaf, second leaf, and whole plants after desubmergence. The leaf and whole plant viability of each genotype was evaluated in the samples shown in **(A)**. Fully green (nonchlorotic) leaves were scored as viable. Plants were scored as viable if a new leaf appeared during recovery. The data represent means ± SD from three independent biological replicates ($n = 75$).

(C) Decrease in chlorophyll *a*/*b* content in leaves during submergence. Fourteen-day-old plants were submerged for up to 14 d, and leaf tissue was harvested at specific time points (days 0, 1, 3, 6, 10, and 14). Chlorophyll was extracted in 80% (v/v) buffered acetone and quantified by a spectrophotometer. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes ($P < 0.05$). FW, fresh weight.

by photosynthesis is restrained as a result of reduced light intensity, CO_2 diffusion, and chlorophyll content. Therefore, long-term submergence causes extensive carbohydrate and energy (nucleotide triphosphate) starvation. To investigate the influence of the *Sub*1 haplotype on carbohydrate metabolism, starch and total soluble carbohydrate contents in leaves were monitored in the two genotypes over 14 d of submergence (Figures 4A and 4B). Leaves of both genotypes had similar starch content (Figure 4A). In M202, starch was rapidly consumed and decreased to 9.8% of the total starch content at day 0 after 3 d of submergence. The decline in starch was significantly less dramatic in M202(*Sub*1), which retained 32.0% of the total starch content on day 3. Nonetheless, starch reserves were greatly depleted in both genotypes by day 10. These observations reveal that M202 leaves experience a more rapid and prolonged period of starch starvation during submergence. A similar trend was confirmed for the amount of total soluble carbohydrates, including glucose, fructose, and sucrose, in leaves of the two genotypes (Figure 4B). More than 60% of soluble carbohydrates were consumed within 1 d of submergence in both genotypes. After the initial response, the consumption rate decelerated, with the magnitude of diminution in total soluble carbohydrates significantly less severe in the tolerant M202(*Sub*1).

A number of metabolic enzymes are involved in the catabolism of starch and soluble carbohydrates in plants. Anoxia-intolerant species, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), fail to degrade conserved starch under conditions of oxygen deprivation because α-amylases are not synthesized and active in their seeds under anoxia (Guglielminetti et al., 1995b). Exceptionally, transcripts of rice α-amylases accumulate in the seed embryo and aleurone during germination even under anoxia (Hwang et al., 1999). In addition, α-amylase protein levels

and activity were shown to be induced by anoxia in rice seedlings (Guglielminetti et al., 1995b). Regulation of leaf α-amylase genes in lowland rice leaves under submergence has not been reported. Semiquantitative RT-PCR detection of the transcripts of three α-amylase genes, *Rice Amylase*-3C (*RAmy*3C), *RAmy*3D, and *RAmy*3E, revealed that their upregulation was controlled by the *Sub*1 locus (Figure 4C). The level of *RAmy*3C mRNA increased immediately under submergence stress, reached a maximum by day 6, and then decreased through day 14 in both genotypes. Overall *RAmy*3C transcript induction was greater in M202. *RAmy*3D and *RAmy*3E transcript increases occurred later than in *RAmy*3C; the increases in these transcripts were considerably lower in M202(*Sub*1) leaves.

Sucrose, which is the major energy source and transport form of carbohydrates in rice, can be hydrolyzed via two distinct pathways: the more energy-efficient sucrose synthase pathway and the invertase pathway (Zeng et al., 1999; Geigenberger, 2003; Fukao and Bailey-Serres, 2004). It has been shown that the transcript level and the enzymatic activity of sucrose synthase increase, whereas those of invertase decrease, during oxygen

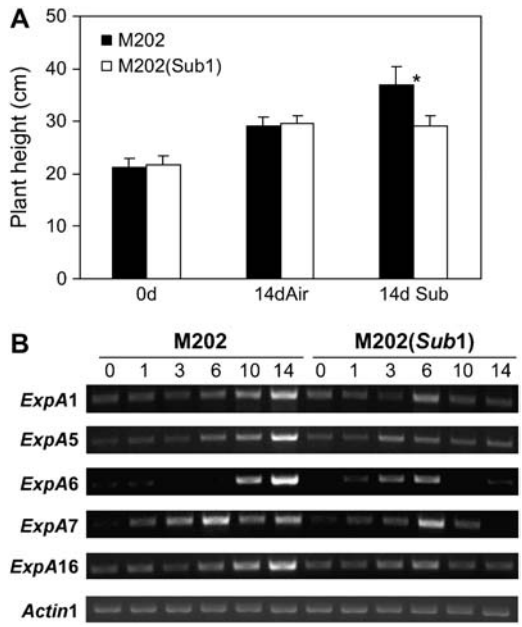

Figure 3. Leaf Elongation in M202 Is Greater Than in M202(*Sub*1) Plants under Submergence.

(A) Plant height after submergence treatment. Fourteen-day-old plants were grown under aerobic (Air) or submerged (Sub) conditions for 14 d. Plant height was measured at days 0 and 14. The height of plants submerged for 14 d was recorded upon desubmergence. The data represent means ± SD from three independent biological replicates ($n = 75$). The asterisk indicates that M202 plants after 14 d of submergence were significantly more elongated than other plants ($P < 0.01$).

(B) Analysis of *ExpA* gene transcript accumulation in leaves during submergence. Fourteen-day-old plants were submerged for up to 14 d, and leaf tissue was collected at specific time points (days 0, 1, 3, 6, 10, and 14). Total RNA was analyzed by semiquantitative RT-PCR using gene-specific primers for *ExpA*. The level of *Actin*1 mRNA was used as a loading control.

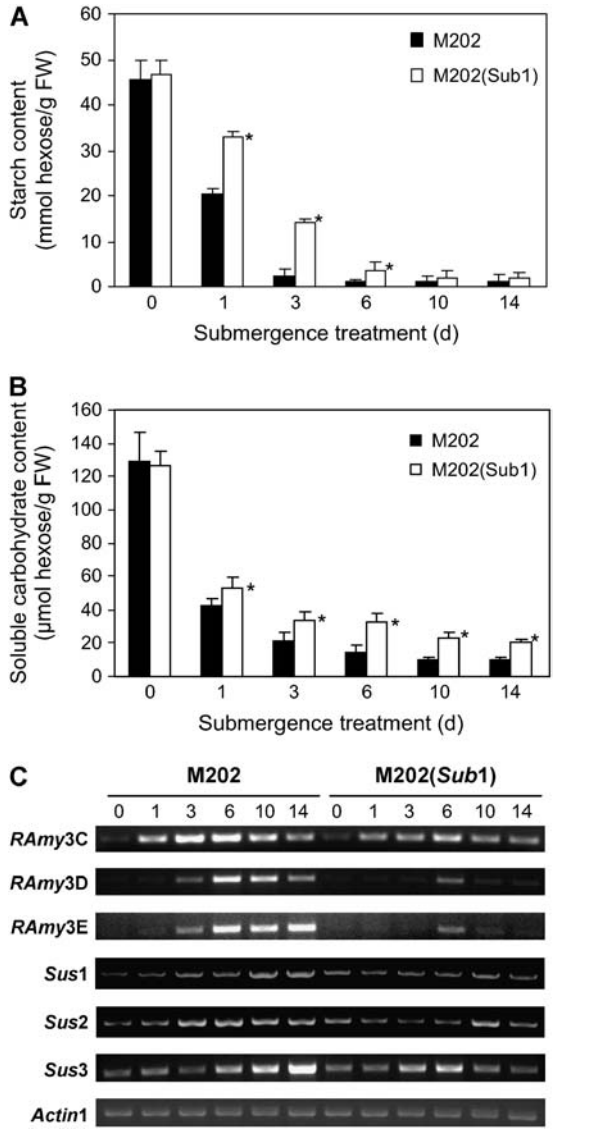

Figure 4. Carbohydrate Consumption Is Accelerated in Leaves of M202 Relative to M202(*Sub*1) during Submergence.

(A) Starch contents in leaves during submergence. Fourteen-day-old plants were submerged for up to 14 d, and leaf samples were collected at specific time points (days 0, 1, 3, 6, 10, and 14). Leaf starch content was determined by an enzymatic method. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.05$). FW, fresh weight.

(B) Total soluble carbohydrate contents in leaves during submergence. Leaf samples used for the starch assay were analyzed to determine total carbohydrate contents by the anthrone method. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.05$).

(C) Accumulation of gene transcripts associated with carbohydrate catabolism. Leaf samples analyzed for starch and total soluble carbohydrates were used to extract total RNA, which was analyzed by semiquantitative RT-PCR using gene-specific primers for α-amylases (*RAmy*) and sucrose synthases (*Sus*). The level of *Actin*1 mRNA was used as a loading control.

deprivation in maize (*Zea mays*) roots and potato (*Solanum tuberosum*) tubers, suggesting that sucrose synthase is the principal enzyme that converts sucrose to phosphorylated hexose sugars under low oxygen stress (Zeng et al., 1999; Geigenberger, 2003). In M202 leaves, the transcript levels of all three sucrose synthase genes, *Sus*1, *Sus*2, and *Sus*3, became increased by the middle of the submergence period and remained increased through day 14 (Figure 4C). By contrast, *Sus* gene transcripts were only transiently and much less dramatically increased in M202(*Sub*1). Thus, the *Sub*1 haplotype controls the regulation of the transcript levels of genes encoding α-amylase and sucrose synthase, which are required for carbohydrate catabolism during submergence.

Transcript Levels of Genes Associated with Ethanolic Fermentation under Submergence Are Controlled by the *Sub*1 Haplotype

The activation of fermentation under conditions of oxygen deprivation is required to recycle NAD^+, which is essential for the maintenance of glycolysis. It is well established that oxygen deprivation generally promotes a transient burst in lactate fermentation that is followed by an extended period of increased ethanolic fermentation (Drew, 1997). This increase typically requires increases in pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). In fact, *adh* and *pdc* loss-of-function mutants in maize, rice, and *Arabidopsis thaliana* succumb rapidly to low oxygen and submergence stress, confirming that ethanolic fermentation is necessary for acclimation to the transient stress (Schwartz, 1969; Rahman et al., 2001; Baxter-Burrell et al., 2003; Ismond et al., 2003; Kürsteiner et al., 2003). To examine the role of the *Sub*1 haplotype in ethanolic fermentation during submergence, transcript levels of the *Pdc* and *Adh* genes were evaluated in leaves of the two genotypes (Figure 5A). In M202, *Pdc*1 mRNA gradually accumulated until day 6 and remained constant until day 14. By contrast, in M202(*Sub*1), the *Pdc*1 mRNA increase was limited. The *Pdc*2 and *Pdc*4 transcript accumulation was consistent with the *Adh*1 and *Adh*2 mRNA levels in both lines. A dramatic increase in these transcripts occurred within 1 d of submergence and remained steady through day 14 in tolerant M202(*Sub*1); the increase of these transcripts was greatly limited in M202.

The enzymatic activities of PDC and ADH were also monitored in leaves of the two genotypes under submergence stress (Figure 5B). PDC activities increased at most 4.9- and 8.1-fold in M202 and M202(*Sub*1), respectively, compared with 14-d-old plants grown in air. The increase in specific activity of PDC was significantly greater in M202(*Sub*1) after day 3. PDC activity increased to a maximum by day 6 and then decreased through day 14 in both genotypes. ADH activities increased 14.8- and 30.6-fold at the maximum in M202 and M202(*Sub*1), respectively. In both genotypes, ADH activity increased continuously for 14 d of submergence, although the rate of increase was gradually lessened. These data suggest that the *Sub*1 haplotype controls the magnitude but not the temporal pattern of PDC and ADH activities during submergence.

The limitation of carbohydrate consumption in the tolerant M202(*Sub*1) could in fact restrict ethanolic fermentation, despite the higher capacity for this process in this line. Ten-day-old seedlings grown on agar medium in test tubes were fully submerged, and ethanol content and ADH activity were monitored at days 0, 1, and 3 (Figures 5C and 5D). Higher ADH activity induction was observed in M202(*Sub*1) but was not reflected by leaf and efflux ethanol content. In M202, the ethanol contents in both the leaves and the surrounding water were significantly higher at day 1 (Figure 5C). At day 3, these values were similar for both genotypes. These data further demonstrate that the *Sub*1 haplotype influences both carbohydrate utilization and the capacity for ethanolic fermentation.

Gene Expression Responsive to Ethylene and GA Is Influenced by the *Sub*1 Haplotype

An increase in the production and accumulation of ethylene is essential for a variety of acclimative responses to submergence (Fukao and Bailey-Serres, 2004). The observation that the intolerant M202 showed increased leaf and internode elongation under submergence led to the hypothesis that the *Sub*1 haplotype might influence production or sensitivity to ethylene. To evaluate this possibility, ethylene released during submergence was measured in the two genotypes (Figure 6A). Ethylene level was enhanced fivefold to sixfold after 1 d of submergence in both genotypes. After 3 d, ethylene evolution continued, but it was 1.6-fold higher in M202 than in M202(*Sub*1).

To discern whether *Sub*1A, *Sub*1B, or *Sub*1C influences the transcript accumulation of ethylene-responsive genes, the transcript levels of these ERF genes and *Sub*1-regulated genes were evaluated in 14-d-old plants after treatment with 1 or 100 ppm ethylene (Figures 6B to 6E). *Sub*1A mRNA, the gene present only in the tolerant line, was increased dramatically in M202(*Sub*1) in response to 1 and 100 ppm ethylene (Figure 6B). Exposure to high levels of ethylene also promoted an increase in the *Sub*1C transcript in both genotypes, but the transcript level was slightly higher in M202. By contrast, *Sub*1B transcript accumulation was not ethylene-responsive. The levels of all expansin transcripts tested increased in response to ethylene in M202 (Figure 6C). However, these genes were either nonresponsive or less responsive to ethylene in M202(*Sub*1), generally consistent with the haplotype-specific differential regulation of these transcripts under submergence. These results suggest that the ethylene- and submergence-responsive induction of *Sub*1A may suppress the expression of the expansin genes.

The ethylene response of genes related to carbohydrate catabolism was also surveyed (Figure 6D). *RAmy*3C and *RAmy*3D mRNA accumulation was responsive to ethylene at similar levels in both genotypes, whereas *RAmy*3E transcript abundance was not affected by ethylene. This result contrasts somewhat with the submergence-promoted increase in *RAmy*3D and *RAmy*3E, which was considerably more pronounced in the M202 genotype. These results indicate an involvement of ethylene that is not repressed by the *Sub*1 haplotype under aerobic conditions. Sucrose synthase gene transcripts were generally increased in response to 1 ppm ethylene, with the exception of *Sus3* mRNA, which remained at the threshold of detection in M202(*Sub*1). Thus, ethylene treatment did not recapitulate the genotype-specific increases in *Sus* transcript levels under submergence.

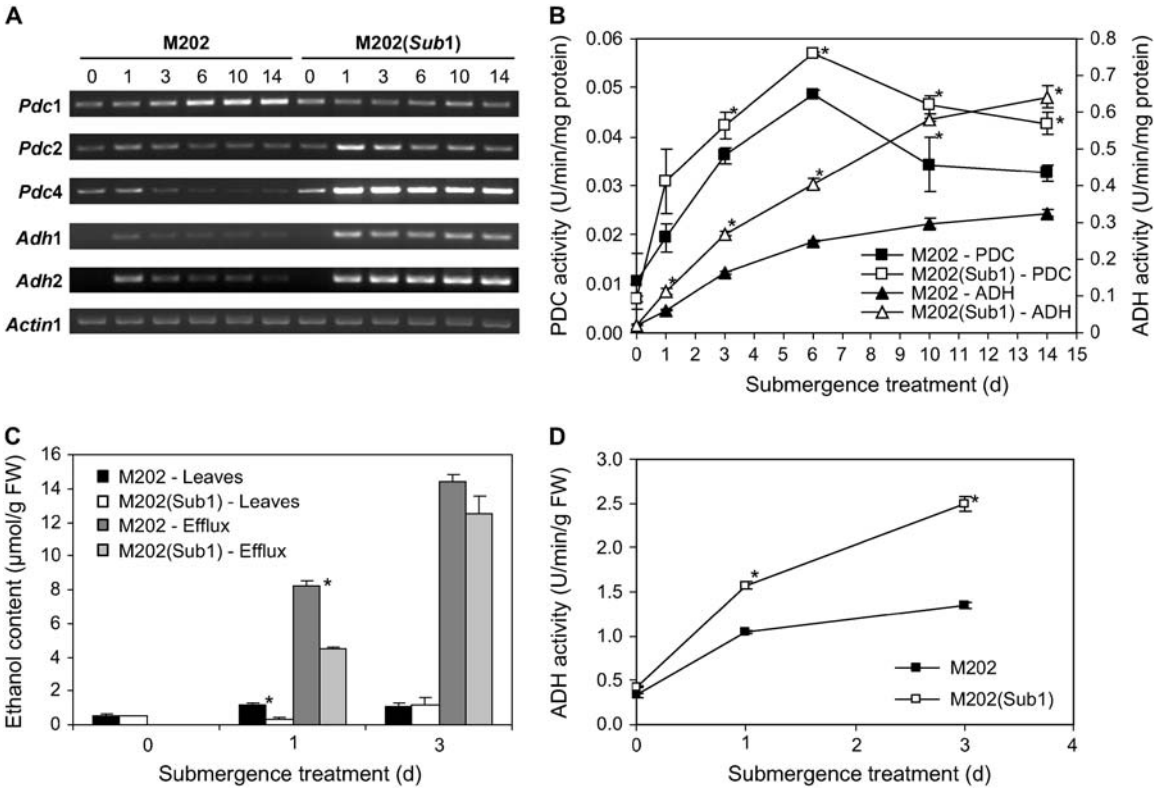

Figure 5. Ethanolic Fermentation in M202 and M202(*Sub*1) Leaves in Response to Submergence.

(A) Accumulation of gene transcripts associated with ethanolic fermentation during submergence. Fourteen-day-old seedlings were exposed to submergence stress for up to 14 d, and leaf tissue was harvested at specific time points (days 0, 1, 3, 6, 10, and 14). Total RNA extracted from the leaf tissues was analyzed by semiquantitative RT-PCR using gene-specific primers for *Pdc* and *Adh* transcripts. The level of *Actin*1 mRNA was used as a loading control.

(B) PDC and ADH activities in leaves during submergence. Specific activities of PDC and ADH were assayed for the leaf tissue used for RT-PCR analysis of *Pdc* and *Adh* mRNAs. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.05$).

(C) Ethanol content of leaves and surrounding medium of submerged plants. Ten-day-old plants were submerged in water in test tubes for up to 3 d. Leaf tissues and the surrounding medium were collected at days 0, 1, and 3, and their ethanol contents were quantified enzymatically. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.01$). FW, fresh weight.

(D) ADH activity in leaves used for the ethanol assay. ADH enzymatic activity was assayed in the leaf tissues used for the analysis of ethanol content. ADH values are presented on a fresh weight basis. The data represent means ± SD from three independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.01$).

Ethylene treatment also altered *Adh* and *Pdc* transcript levels (Figure 6E). Low and high concentrations induced an obvious accumulation of *Pdc*1 transcript in M202 but not in M202(*Sub*1). Conversely, the ethylene-mediated increase in the other mRNAs associated with ethanolic fermentation, such as *Pdc*2, *Pdc*4, *Adh*1, and *Adh*2, was restrained in M202 compared with M202(*Sub*1). The distinct ethylene-responsiveness of the genes related to ethanolic fermentation in the two genotypes corresponded directly with expression patterns observed during submergence (Figure 5A).

GA is also implicated in the acclimative response to submergence, particularly the elongation of internodes and petioles during submergence in deepwater rice and *R. palustris*, respectively (Kende et al., 1998; Peeters et al., 2002). A number of expansin genes are upregulated by GA in the internodes of deepwater rice (Cho and Kende, 1997; Lee and Kende, 2002). GA also induces the mRNA accumulation of some α-amylase genes in the embryo and aleurone layers of cereal seeds (Bethke et al., 1997; Gibson, 2004). To discern any effect of the *Sub*1 haplotype on the response to GA in leaves, we evaluated the expression of the three *Sub*1 ERFs and the GA-responsive genes associated with acclimation to submergence stress (Figure 7A). The transcript levels of *Sub*1A and *Sub*1B were unaltered by 5 and 50 μM GA_3, whereas *Sub*1C mRNA accumulation was induced by GA treatment, specifically in M202. This GA-responsiveness correlated with higher levels of *Sub*1C under submergence in M202. *RAmy*3C mRNA, which is responsive to GA, accumulated similarly in both genotypes after GA treatment. By contrast, the

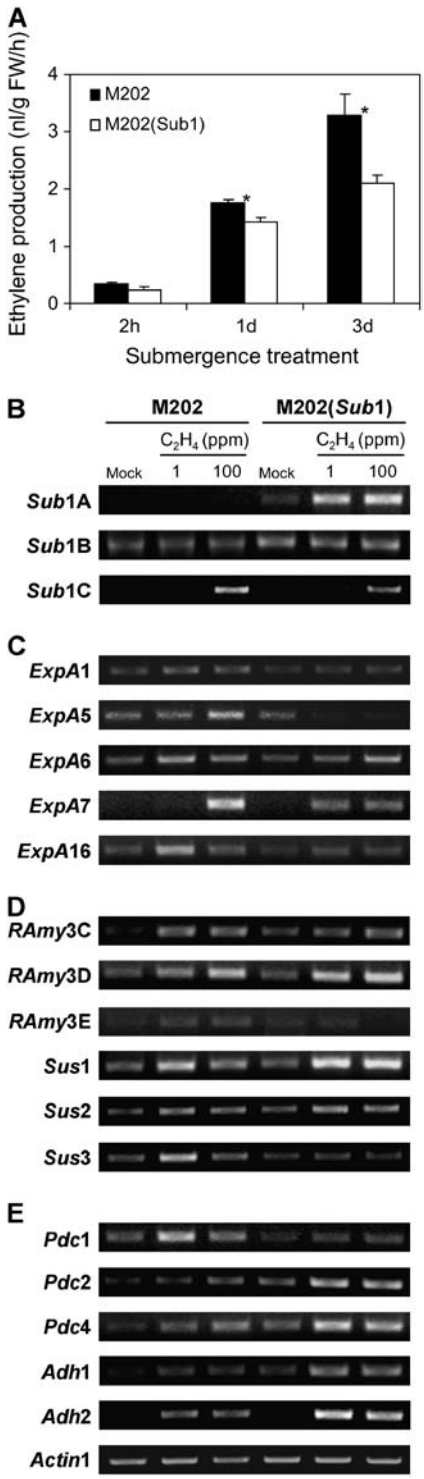

Figure 6. Ethylene Sensitivity Is Greater in Leaves of M202 Relative to M202(*Sub*1).

(A) Ethylene production during submergence. Ten-day-old plants were submerged in water in test tubes for up to 3 d. Ethylene gas that accumulated in the headspace of the test tube was quantified by gas chromatography. The data represent means ± SE from five independent biological replicates. Asterisks indicate significant differences between the two genotypes at that time point ($P < 0.05$). FW, fresh weight.

(B) Analysis of *Sub*1 region gene transcript accumulation in response to ethylene. Fourteen-day-old plants were treated with 1 or 100 ppm ethylene for 6 h in the light. Total RNA was analyzed by semiquantitative RT-PCR using gene-specific primers for *Sub*1A, *Sub*1B, and *Sub*1C, as described for Figure 1B.

(C) Analysis of *ExpA* gene transcript accumulation in response to ethylene. Total RNA extracted from ethylene-treated leaves was analyzed by semiquantitative RT-PCR using gene-specific primers for *ExpA*, as described for Figure 3.

(D) Analysis of transcript levels for genes associated with carbohydrate metabolism in response to ethylene. Total RNA extracted from ethylene-treated leaves was analyzed by semiquantitative RT-PCR using gene-specific primers for α-amylases (*RAmy*) and sucrose synthases (*Sus*), as described for Figure 4.

(E) Analysis of transcript levels for genes associated with ethanolic fermentation in response to ethylene. Total RNA extracted from ethylene-treated leaves was analyzed by semiquantitative RT-PCR using gene-specific primers for *Pdc* and *Adh*, as described for Figure 5.

The level of *Actin*1 mRNA was used as a loading control for **(B)** to **(E)**.

induction of *RAmy*3D mRNA was much greater in M202 with 50 μM GA_3. Of the five expansins analyzed here, only *ExpA*1 and *ExpA*7 transcripts accumulated after GA treatment, but a clear difference was not observed between the two genotypes (Figure 7A). GA-promoted elongation under normal growth conditions was similar in 14-d-old seedlings of the two genotypes (Figure 7B), consistent with the similar accumulation of *ExpA* transcripts in response to GA_3.

DISCUSSION

The results presented here demonstrate that the *Sub*1 haplotype regulates diverse acclimative responses to submergence, including chlorophyll breakdown, leaf and internode elongation, and carbohydrate catabolism, as well as the induction of enzymes required for ethanolic fermentation. Nearly 100% of M202(*Sub*1) plants with the *Sub*1 haplotype from FR13A (*Sub*1A-1, *Sub*1B-1, and *Sub*1C-1) recovered from 14 d of submergence compared with <40% of M202 plants (*Sub*1B-2 and *Sub*1C-2). The management of carbohydrate and thereby energy economy is critical to surviving oxygen deprivation, as the consequential ATP shortfall leads to a reduction in cytosolic pH and a loss of membrane integrity (Greenway and Gibbs, 2003). In general, the amount of stored carbohydrates in plant organs is positively correlated with the level of submergence tolerance (Jackson and Ram, 2003). Consistently, starch and soluble carbohydrates were better preserved in M202(*Sub*1) leaves during submergence (Figures 4A and 4B). The observed distinctions in α-amylase and sucrose synthase mRNA accumulation are likely to be responsible for the repression of carbohydrate consumption in M202(*Sub*1) leaves (Figure 4C). The better maintenance of chlorophyll in M202(*Sub*1) leaves would lessen carbohydrate starvation, because photosynthesis may continue even under submerged conditions, albeit at low levels as a result of the reduced light intensity and CO_2 availability (Figure 2C). Indeed, anatomical and biochemical adjustments to submergence, such

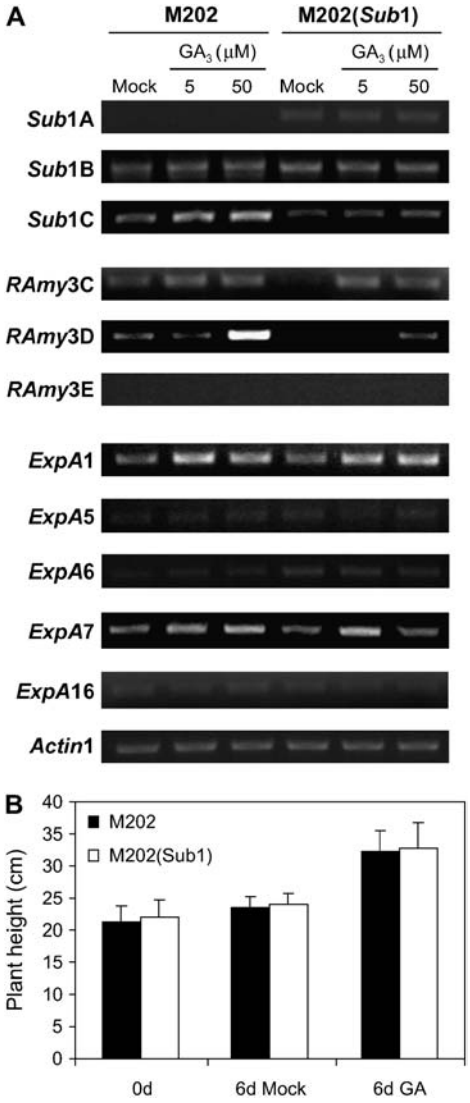

Figure 7. GA_3 Sensitivity of M202 and M202(*Sub*1) Leaves.

(A) Analysis of transcript levels for *Sub*1 region, α-amylase (*RAmy*), and *ExpA* genes in response to GA_3. Leaves of 14-d-old plants were treated with 5 or 50 μM GA_3 for 24 h. Total RNA extracted from GA_3-treated leaves was analyzed by semiquantitative RT-PCR using gene-specific primers for *Sub*1 genes, α-amylase, and *ExpA*. The level of *Actin1* mRNA was used as a loading control.

(B) Response of plant growth to GA_3. Five- or 14-d-old plants were treated with mock solution (0.1% [v/v] DMSO) or 100 μM GA_3 solution in 0.1% (v/v) DMSO for 3 d. Plant height in the two genotypes was measured after 3 d of treatment. The data represent means ± SD from three independent biological replicates ($n = 75$).

as reduction of gas diffusion resistance by thinning of the cell wall and cuticle, restriction of photorespiration, and attenuation of excitation pressure on the chloroplast electron transport system in leaves, contribute to the continued photosynthesis during submergence in submergence-tolerant *R. palustris* (Mommer et al., 2005). The greater tolerance of the coleoptile to damage than the first and second leaves is in keeping with the observation that the coleoptile emerges from the seed of rice and *Echinochloa* species during germination under anaerobic conditions as a result of the ability to use starch reserves of the seed, whereas the emergence of leaves is more sensitive to the availability of oxygen (Kennedy et al., 1980).

A major function of ethanolic fermentation during submergence is to recycle NAD^+ from NADH, which is required for anaerobic energy production by glycolysis (Fukao and Bailey-Serres, 2004). Paradoxically, the FR13A *Sub*1 haplotype limited starch and soluble carbohydrate depletion during submergence but increased PDC and ADH activities, which enhance the capacity for ethanolic fermentation (Figures 4 and 5). Interestingly, PDC and ADH activities were not positively correlated with the depletion of carbohydrate reserves or ethanol production during submergence. The amount of endogenous and effluxed ethanol was higher for M202 at day 1 of submergence, even though the enzymatic activity of ADH was lower (Figures 5C and 5D). Despite the dichotomy in gene regulation, the production of ethanol was not markedly different by day 3 of submergence in the two lines. The benefit of increased ADH might extend beyond the regeneration of NAD^+. It is thought that the accumulation of acetaldehyde results in serious cellular injury during oxygen deficiency (Drew, 1997; Gibbs and Greenway, 2003). Indeed, leaves of submergence-tolerant rice varieties contained lower levels of aldehydes during submergence (Singh et al., 2001). It is possible that the more dramatic induction of ADH during submergence in M202(*Sub*1) is critical for the timely conversion of the toxic byproduct, acetaldehyde, to neutral and diffusable ethanol.

Submergence stimulates ethylene production and accumulation within plant tissues (Banga et al., 1996; Kende et al., 1998). Ethylene evolution increased considerably in the two rice genotypes studied, with significantly greater levels in M202 than in M202(*Sub*1) after 3 d of submergence, indicating another role of the *Sub*1 haplotype (Figure 6A). Although ethylene plays a central role in adaptive responses to submergence, excessive ethylene accumulation may cause negative responses that compromise submergence tolerance. In *Citrus* fruit, ethylene promotes gene expression, de novo protein synthesis, and enzymatic activity of chlorophyllase (Trebitsh et al., 1993; Jacob-Wilk et al., 1999). Application of an ethylene biosynthesis/signaling inhibitor, 1-methylcyclopropene, reduced chlorophyllase gene expression, enzymatic activity, and chlorophyll breakdown in rice leaves during submergence (Ella et al., 2003). This treatment also suppressed the consumption of starch and soluble sugars during the stress and enhanced recovery. Thus, ethylene enhances the enzymatic degradation of chlorophyll and the consumption of carbohydrate reserves. The finding that the *Sub*1 haplotype influences ethylene production and the expression of ethylene-responsive genes strongly suggests that the *Sub*1 locus regulates production and sensitivity to ethylene, which finely modulates myriad acclimative responses to submergence, including leaf and internode elongation, carbohydrate consumption, and ethanolic fermentation.

The *Sub*1 locus is polymorphic and can encode two or three submergence-inducible ERF genes, of which all or a subset could regulate the expression of genes associated with acclimation to submergence. The accumulation of *Sub*1A and *Sub*1B transcripts

was more pronounced in M202(*Sub*1), whereas *Sub*1C was more highly induced in M202, throughout the period of submergence (Figure 1B). Several lines of evidence indicate that *Sub*1A confers submergence tolerance (Xu et al., 2006). First, this gene is absent in all *japonica* and some *indica* varieties, all of which are submergence-intolerant. Second, *indica* lines carrying a *Sub*1A allele with a Ser-to-Pro substitution at residue 186 and showing a lower level of induction are intolerant to submergence. Finally, transformation of an intolerant *japonica* rice variety with a maize *ubiquitin1* promoter:*Sub*1A-1 (*Ubi1:Sub*1A-1) displayed increased submergence tolerance. The demonstration here of the differential regulation of *Sub*1 region genes by ethylene and GA provides further evidence that *Sub*1A-1 is the critical determinant in submergence tolerance provided by the FR13A haplotype. The low level of expression of *Sub*1C-1 in response to submergence, ethylene, and GA could be attributable to distinctions in the promoter of this allele and/or negative regulation of *Sub*1C-1 by *Sub*1A-1. *Sub*1A and *Sub*1C transcripts are increased by the application of ethylene, with slightly higher induction of *Sub*1C mRNA in M202 (Figure 6B). The distinctions in ethylene-induced transcript levels in the two lines show that *Sub*1A directly or indirectly affects the activation and repression of a subset of the ethylene-responsive genes evaluated here. We favor the conclusion that SUB1A-1 negatively regulates *Sub*1C expression because of two independent observations. First, the level of *Sub*1A transcript is extremely low during submergence, but *Sub*1C mRNA is highly accumulated under the stress in intolerant *indica* lines. Second, transgenic lines that ectopically express *Sub*1A-1 (*Ubi*1:*Sub*1A-1) in *japonica* rice display limited induction of *Sub*1C-2 under submergence (Xu et al., 2006). These transgenic lines are significantly reduced in stature at the seedling and adult stages (Xu et al., 2006; T. Fukao and K. Xu, unpublished data). In *Arabidopsis*, overexpression of *Arabidopsis ERF*1 and tomato (*Solanum lycopersicum*) *Pti*4 also decreases plant size in a manner similar to the constitutive ethylene response mutant *constitutive triple response1* and *Ethylene-Insensitive3*/*Ethylene-Insensitive-like1*–overexpressing plants (Solano et al., 1998; Gu et al., 2002; Wu et al., 2002). These observations further support the notion that SUB1A, a putative DNA binding protein, regulates ethylene-responsive gene expression.

The *Sub*1 haplotype also influences GA-mediated gene expression. Application of GA_3 promoted the accumulation of *Sub*1C transcript in leaves of M202 but not in M202(*Sub*1) (Figure 7). GA treatment increased the levels of *RAmy*3D transcript only in M202. This finding indicates that in the absence of *Sub*1A-1, *Sub*1C may function to promote *RAmy*3D mRNA accumulation in response to GA. The transcription and transcript stability of genes associated with carbohydrate catabolism are regulated by sugar availability. Sucrose starvation prolonged the half-lives of *RAmy*1A, *RAmy*3D, and *RAmy*3E in rice suspension cells (Sheu et al., 1996). Application of exogenous sugars, such as glucose, fructose, and sucrose, suppresses α-amylase expression at the transcriptional and posttranscriptional levels in cereal seeds (Loreti et al., 2000; Gibson, 2004). In maize root tips, the level of hypoxia-inducible sucrose synthase transcript is repressed by glucose (Koch, 2004). Interestingly, exogenous glucose and sucrose repress GA-mediated α-amylase expression in barley embryos (Perata et al., 1997; Loreti et al., 2000). These data suggest that the alteration of carbohydrate content during submergence would affect the expression of genes responsive to sugar and GA. Distinctions in levels of α-amylase and sucrose synthase transcripts in the two genotypes during submergence could reflect significant differences in soluble carbohydrate reserves in leaves. The *Sub*1 haplotype might reduce carbohydrate responsiveness, because soluble carbohydrates in M202(*Sub*1) decrease to levels lower than those that induce α-amylase and sucrose synthase transcripts in M202 during submergence.

We propose a model for the ethylene- and GA-mediated transcriptional regulation of genes involved in acclimation to submergence by SUB1A and SUB1C (Figure 8). Submergence-promoted ethylene production or entrapment stimulates the accumulation of the *Sub*1A transcript in leaves of submergence-tolerant rice.

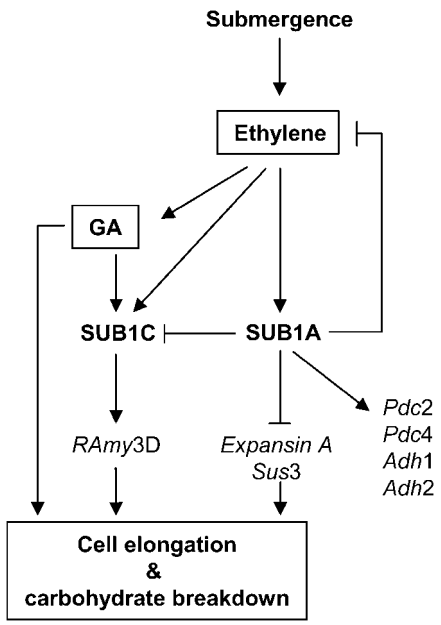

Figure 8. Model for Ethylene- and GA-Mediated Regulation of Gene Transcripts Associated with Acclimative Responses to Submergence by SUB1A and SUB1C.

Submergence triggers ethylene production and accumulation within plant cells, which promotes the accumulation of *Sub*1A and *Sub*1C transcripts. SUB1A of submergence-tolerant *indica* activates the expression of genes associated with ethanolic fermentation and represses the expression of genes involved in cell elongation and carbohydrate breakage. SUB1A also limits the production of ethylene during submergence, which restricts GA production and sensitivity. Repression of ethylene-mediated GA production and response results in the restriction of *Sub*1C mRNA accumulation as well as GA-dependent cell elongation and carbohydrate consumption. SUB1A also suppresses the accumulation of *Sub*1C transcript during submergence. A limitation of GA-dependent carbohydrate consumption by SUB1A may suppress α-amylase and sucrose synthase mRNA accumulation, because transcription of these genes is enhanced by sugar starvation. Consequently, SUB1A of submergence-tolerant *indica* regulates the ethylene- and GA-mediated gene expression responsible for carbohydrate consumption, cell elongation, and ethanolic fermentation and thereby confers submergence tolerance in lowland rice.

SUB1A of tolerant *indica* enhances the levels of mRNAs associated with ethanolic fermentation and represses the accumulation of mRNAs responsible for cell elongation and carbohydrate catabolism. SUB1A also restricts ethylene production during submergence, possibly by feedback regulation. It has been shown that ethylene increases the endogenous GA content and sensitivity to this phytohormone in deepwater rice and *R. palustris* (Kende et al., 1998; Peeters et al., 2002). Although the level of *Sub*1C mRNA is increased by both ethylene and GA, the presence of *Sub*1A is correlated with limited accumulation of *Sub*1C mRNA. This suppression of *Sub*1C mRNA accumulation is also observed during submergence in M202(*Sub*1) and transgenic lines overexpressing *Sub*1A (Xu et al., 2006). It follows that through limitation of ethylene-mediated GA production and subsequent *Sub*1C mRNA accumulation, SUB1A represses GA-mediated responses in cell elongation and carbohydrate breakdown. The level of soluble carbohydrates in M202(*Sub*1) may be maintained above the threshold that promotes the transcription of genes responsive to sugar starvation, including α-amylases and sucrose synthases. Thus, the *Sub*1A-1 allele of tolerant *indica* is responsible for the repression of ethylene- and GA-mediated gene expression involved in carbohydrate consumption and cell elongation as well as the enhancement of fermentation capacity during submergence. The coordination of carbohydrate consumption and energy economy by *Sub*1A influences the enhancement of submergence tolerance in lowland rice. The analysis of the molecular functions of the three SUB1 proteins is under investigation.

METHODS

Plant Materials and Growth Conditions

Rice (*Oryza sativa*) cv M202 and the *Sub*1 introgression line M202(*Sub*1) (accession number DX236-17-2-4) were kindly provided by Abdelbagi Ismail and David Mackill of the International Rice Research Institute (Xu et al., 2004). The seeds were sterilized in 1% (v/v) sodium hypochlorite for 1 h and rinsed thoroughly with deionized water. Seeds were placed on wet filter paper for 3 d at 25°C in the dark, and pregerminated seeds were transplanted into soil-containing pots and grown for 14 d in a greenhouse (30°C day, 20°C night) under natural light conditions.

Submergence, Ethylene, and GA Treatments

All submergence and hormonal treatments were replicated in at least three independent biological experiments. Light gray plastic tanks (65 × 65 × 95 cm) were filled with 90 cm of water, which was left standing for 1 d in the greenhouse (30°C day, 20°C night) before placement of the potted plants in the tank. Fourteen-day-old seedlings in soil-containing pots were completely submerged for up to 14 d. The tank water was not circulated or refreshed during the treatment. The turbidity of the tank water did not visibly increase during the submergence period. All leaves of each plant were harvested at 3 PM on the day of treatment specified, immediately frozen in liquid nitrogen, and stored at −80°C until use. Whole plant and leaf viability was evaluated after 7 d of recovery under normal growth conditions. Plants were scored as viable when one or more new leaves appeared during the recovery period. The viability of the coleoptile, first leaf, and second leaf was also recorded after recovery. Fully green (nonchlorotic) leaves were scored as viable.

Fourteen-day-old plants were treated with ethylene gas or GA_3 solution before gene expression analysis. For ethylene treatment, seedlings were placed in a closed Lucite chamber filled with 1 or 100 ppm ethylene in air for 6 h in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). For GA treatment, leaves from 14-d-old plants were floated on mock solution (0.05% [v/v] DMSO) or GA_3 solution containing 5 or 50 μM GA_3 (Sigma-Aldrich) and 0.05% (v/v) DMSO for 24 h in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After treatment, leaf samples were frozen immediately in liquid nitrogen and stored at −80°C until use.

To observe the response of plant growth to GA, sterilized seeds were incubated on wet filter paper for 5 d in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and the seedlings were transferred to mock solution (0.1% [v/v] DMSO) or 100 μM GA_3 solution in 0.1% (v/v) DMSO. Fourteen-day-old seedlings (25 plants) were sprayed with mock solution or 200 mL of GA_3 solution (100 μM) once per day for 3 d in the greenhouse. Plant height of each seedling (8 or 17 d old) was recorded after 3 d of treatment.

Ethylene Measurement

The relative rate of ethylene production was measured during submergence. Dehulled seeds were sterilized in 70% (v/v) ethanol for 10 min and in 2% (v/v) sodium hypochlorite for 20 min. After rinsing with sterilized deionized water, each seed was placed on 1× Murashige and Skoog medium (3 mL) in a 16-mm × 15-cm test tube at 20°C for 10 d (16 h light/8 h dark; light level, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). For submergence stress, the test tube was filled with 18 mL of sterilized deionized water, closed with a loose plastic cap, and incubated for up to 3 d at 25°C in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Two hours before the submergence treatment was complete, the test tube was tightly closed with a rubber serum stopper, and 2 h later, the accumulated gas sample (0.9 mL) was withdrawn from the headspace of the test tube with a 1-mL syringe and analyzed using a gas chromatograph (6850 series; Hewlett Packard) equipped with an alumina-based capillary column (Agilent Technologies) (Larsen and Cancel, 2004).

Semiquantitative RT-PCR

Total RNA was extracted from 100 mg of tissue using the RNeasy plant mini kit (Qiagen). Single-stranded cDNA was synthesized from 2 μg of total RNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed in a reaction mixture (50 μL) containing 2 μL of cDNA solution, 5 μL of 10× PCR buffer, 0.2 μM primers, 0.2 mM deoxynucleotide triphosphates, and 1.25 units of Taq DNA polymerase (Qiagen). Sequences of primer pairs, PCR conditions, and product sizes are listed in Supplemental Table 1 online. The number of cycles used for amplification with each primer pair was adjusted to be in the linear range. RT-PCR products were confirmed by DNA sequence analysis. All RT-PCR data are representative of at least three independent experiments replicated over the course of several months.

Chlorophyll Assay

Chlorophyll *a*/*b* contents were assayed as described by Porra et al. (1989). Chlorophyll was extracted from 50 mg of tissue in 1 mL of 80% (v/v) acetone containing 2.5 mM sodium phosphate buffer, pH 7.8, on ice. After centrifugation at 4°C for 20 min at 16,000*g*, $A_{663.6}$, $A_{646.6}$, and A_{750} were measured with a spectrophotometer (DU800; Beckman).

Carbohydrate Assay

Leaf tissue (50 mg) was homogenized in 1 mL of 80% (v/v) ethanol and incubated at 80°C for 20 min. After centrifugation at 16,000*g* for 10 min, the supernatant was collected in a new tube. The tissue extraction procedure was repeated twice. The three extracts were combined, dried under vacuum, and dissolved in 0.5 mL of water. Total soluble carbohydrate content was quantified by the anthrone method, with glucose as the standard (Yem and Willis, 1954). The sample solution (100 μL) was mixed with 1.0 mL of 0.14% (w/v) anthrone solution in 100% H_2SO_4 and incubated in

boiling water for 20 min. After cooling, A_{620} was determined. Starch content was measured according to the method of Walters et al. (2004). The ethanol-insoluble fraction was washed with water, resuspended in 0.5 mL of water, and autoclaved at 121°C for 3 h. After cooling, the suspension was adjusted to 25 mM sodium citrate, pH 4.8, and hydrolyzed with 9 units of α-amylase and 3 units of amyloglucosidase for 16 h at 37°C. The glucose content was assayed enzymatically by the method of Guglielminetti et al. (1995a).

Ethanol Assay

Ten-day-old seedlings were submerged in test tubes for up to 3 d as described for the ethylene measurements. Ethanol content in leaves and the water used for seedling submergence was quantified by the method of Rumpho and Kennedy (1981). Leaf tissue (20 mg) was homogenized in 0.2 mL of 5 M ice-cold $HClO_4$, and the extract was centrifuged at 4°C for 20 min at 16,000*g*. The supernatant (100 μL) was neutralized with 0.25 mL of 1 M K_2CO_3, and the precipitates were removed by centrifugation. The obtained supernatant and the water used for submergence were analyzed for ethanol in an assay mixture (1 mL) containing 100 μL of sample solution, 100 mM Tris-HCl, pH 9.0, 0.6 mM NAD^+, and 40 units of ADH. The samples were incubated at 25°C for 1 h, and ethanol content was determined by measuring the reduction of NAD^+ at A_{340}. Ethanol was used as the standard.

Enzyme Activity Assay

PDC (EC 4.1.1.1) activity was assayed as described by Fukao et al. (2003). Crude protein was extracted from 50 mg of tissue in ice-cold extraction buffer (0.4 mL) containing 50 mM MEPS-NaOH, pH 6.2, 1 mM $MgCl_2$, 5 mM DTT, and 1 mM thiamine pyrophosphate chloride. The extract was centrifuged at 4°C for 20 min at 16,000*g*. The reaction mixture (1 mL) contained 100 μL of extract, 50 mM MEPS-NaOH, pH 6.2, 0.5 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate chloride, 10 mM oxamate, 0.12 mM NADH, and 660 nkat of yeast ADH. To initiate the reaction, 50 μL of 400 mM pyruvate was added, and the coupled NADH oxidation was monitored at A_{340} at 25°C for 2 min. ADH (EC 1.1.1.1) activity was assayed by the method of Fukao et al. (2003). Leaf tissue (50 mg) was homogenized in cold extraction buffer (0.4 mL) containing 100 mM Tris-HCl, pH 9.0, 20 mM $MgCl_2$, and 0.1% (v/v) 2-mercaptoethanol on ice. After centrifugation, the supernatant was used for the activity assay. The reaction mixture (1 mL) contained 50 μL of extract, 50 mM Tris-HCl, pH 9.0, and 1 mM NAD^+. Ethanol (50 μL) was added to initiate the reaction, and NAD^+ reduction was monitored at A_{340} at 25°C for 2 min. Protein was quantified by the method of Bradford (1976), with BSA as the standard.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ011598 (*Sub*1A), AP005705 (*Sub*1B), and AP006758 (*Sub*1C).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. Sequences of Primers and PCR Conditions Applied for Semiquantitative RT-PCR.

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